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Necrosis as Programmed Cell Death

Ma. Luisa Escobar, Olga M. Echeverría and Gerardo H. Vázquez-Nin

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Abstract

The process of cell death is the mechanism through which organisms eliminate useless cells. Hence, it is a normal process that maintains homeostasis. Cell removal can be effected by several pathways that involve complex and regulated molecular events specific to each type of cell death. Diverse studies have evidenced different types of cell death: apoptosis, autophagy, and necrosis. This chapter presents a brief review of the apoptotic and autophagic cell death processes but focuses attention primarily on necrosis because it has previously been considered an accidental and uncontrolled form of cell death. More recent evidence, however, has shown that, under certain circumstances, necrosis is conducted by a controlled program called necroptosis, which is now included as a programmed cell death process.

Keywords: Apoptosis, autophagy, cell death, necrosis, necroptosis

1. Introduction

The tissular environment includes a series of signals that maintain the rates of cell proliferation and cell death so as to conserve structural integrity and functionality. Alterations in either one of these processes can cause certain pathologies, such as cancer. The cell death process is an ongoing event during the development of tissues and organs, one that is present right from embryonic development in the form of programmed cell death, which occurs under physiological conditions as a process that requires the active participation of highly regulated mechanisms. Traditionally, apoptosis was synonymous with programmed cell death; however, different routes of cell death, such as autophagy and, more recently, necroptosis, are now included as forms of programmed cell death. Morphologically, each one of these cell death processes has features that make it possible to distinguish among them. The different molecular mechanisms involved in the cell death pathways are responsible for the morphological changes that occur in the affected cells. However, each pathway has specific characteristics;

for instance, cellular shrinkage is a phenomenon that occurs in apoptosis [1], but is not present in other types of cell death, such as autophagy or necrosis. On the other hand, the extensive presence of vesicles evidences autophagy but does not appear to the same extent in the other types of cell elimination [2]. Necrosis, meanwhile, presents generalized swelling of membranous organelles that leads to cell rupture [3].

2. Brief description of two types of programmed cell death: apoptosis and autophagy

Apoptosis, or type I programmed cell death, is the most widely studied of the forms of cell death. Its morphological characteristics can be identified under light microscopy, and include cell shrinkage, compacting of the chromatin, blebbing of the cytoplasmic membrane, and, finally, the formation of apoptotic bodies [1] (Figure 1). Biochemically, apoptosis is characterized by the participation of proteases called caspases, orderly internucleosomal DNA fragmentation, phosphatidylserine externalization, changes in mitochondrial membrane permeability, and the participation of members of the Bcl-2 protein family.

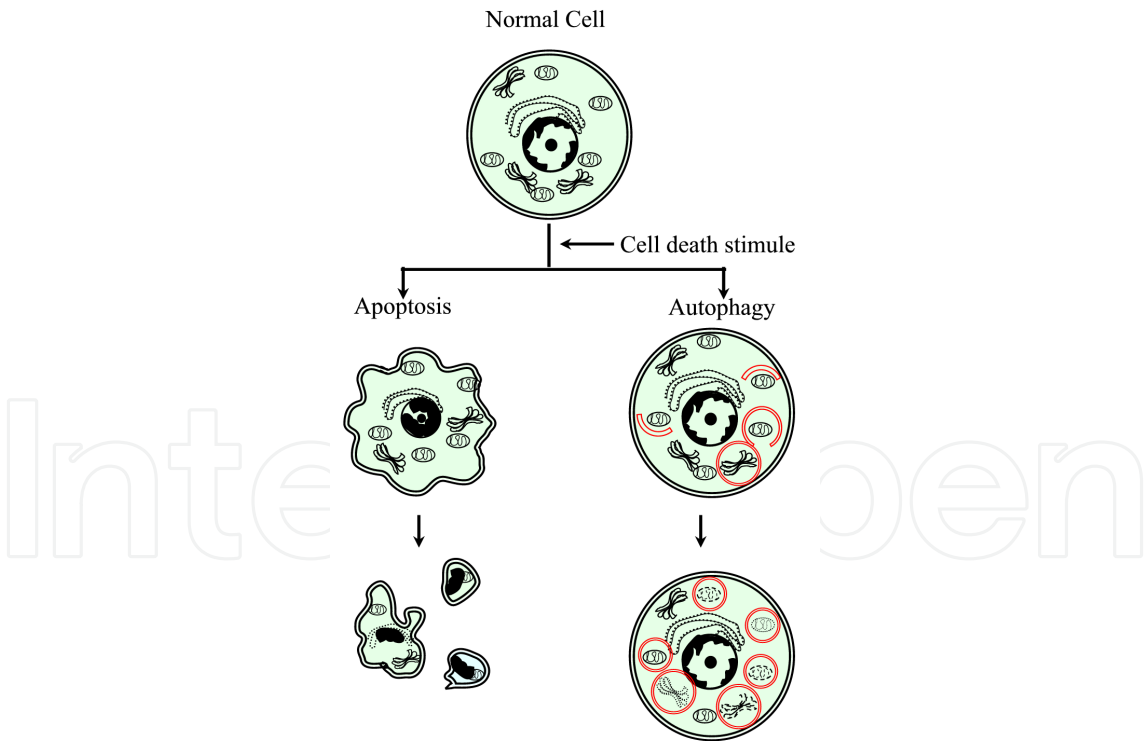


Figure 1. Schematic representation of the programmed cell death process type I (apoptosis) and type II (autophagy). Apoptosis is characterized by a cellular contraction, chromatin compaction, membranous blebs, and the formation of apoptotic bodies. Autophagy is characterized by the presence of a large number of autophagosomes with cytoplasmic content. Both types of cell death do not generate an inflammatory response since the cytoplasmic membrane is conserved until the cellular debris are eliminated by neighborhood or by specialized ones.

Caspases are cysteinyl-aspartate-specific proteases that are synthesized in an inactive form as zymogens called pro-caspases (Figure 2). It is this inactive form that allows the controlled execution of the cell death process. Caspases were first identified in the nematode *Ceanorhabditis elegans* [4], but homologous forms are present in mammals [5].

The hallmarks of apoptosis, such as DNA fragmentation and compacted chromatin, result from caspase activity. During apoptosis, DNA is fragmented into nucleosome size (200 bp) [6, 7]. The factor responsible for DNA fragmentation during apoptosis is a specific DNase (CAD, caspase-activated DNase) that is activated by active caspase-3 [8]. Active caspase-3, in turn, is involved in morphological cell changes during apoptosis, where it cleaves rho-associated kinase-1 (ROCK-1) in order to activate it and this, finally, affects the cytoskeletal arrangement causing the apoptotic shrinkage morphology [9].

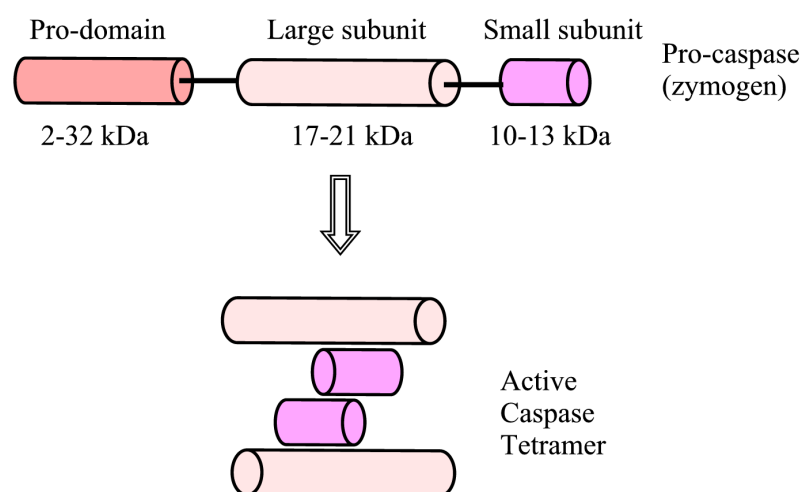


Figure 2. Caspases involved in the apoptotic process are synthesized in an inactive form as zymogens, are constituted by a pro-domain, a large subunit, and a small subunit. The zymogens are activated forming tetramers.

Apoptotic cell death is highly regulated by members of the B-cell lymphoma 2 (Bcl-2) family [10]. Bcl-2 family members have been classified as anti-apoptotic and pro-apoptotic proteins according to their Bcl-2 homology (BH) and domain organization (Figure 3). The presence of domains BH1, BH2, BH3, and BH4 corresponds to the group that inhibits apoptosis. The pro-apoptotic group, in contrast, is divided in two groups: those with domains BH1, BH2, and BH3, and those with only the BH3 domains (defined as BH3 only; see the review in [11]). This family of proteins performs its functions at the intracellular level inside the mitochondria, a key element in apoptosis.

Apoptosis can be initiated by two well-described routes: the extrinsic and intrinsic pathways (Figure 4). Extrinsic activation is conducted through the participation of death ligands (such as the tumor necrosis factor – TNF – superfamily, and TNF-related apoptosis-induced ligands, or TRAIL) with their cognate cell surface death receptors (such as TNF receptor 1, Fas, TRAIL receptor 1, or TRAIL receptor 2) (reviewed in [12]). Once the ligand recognizes and bonds to

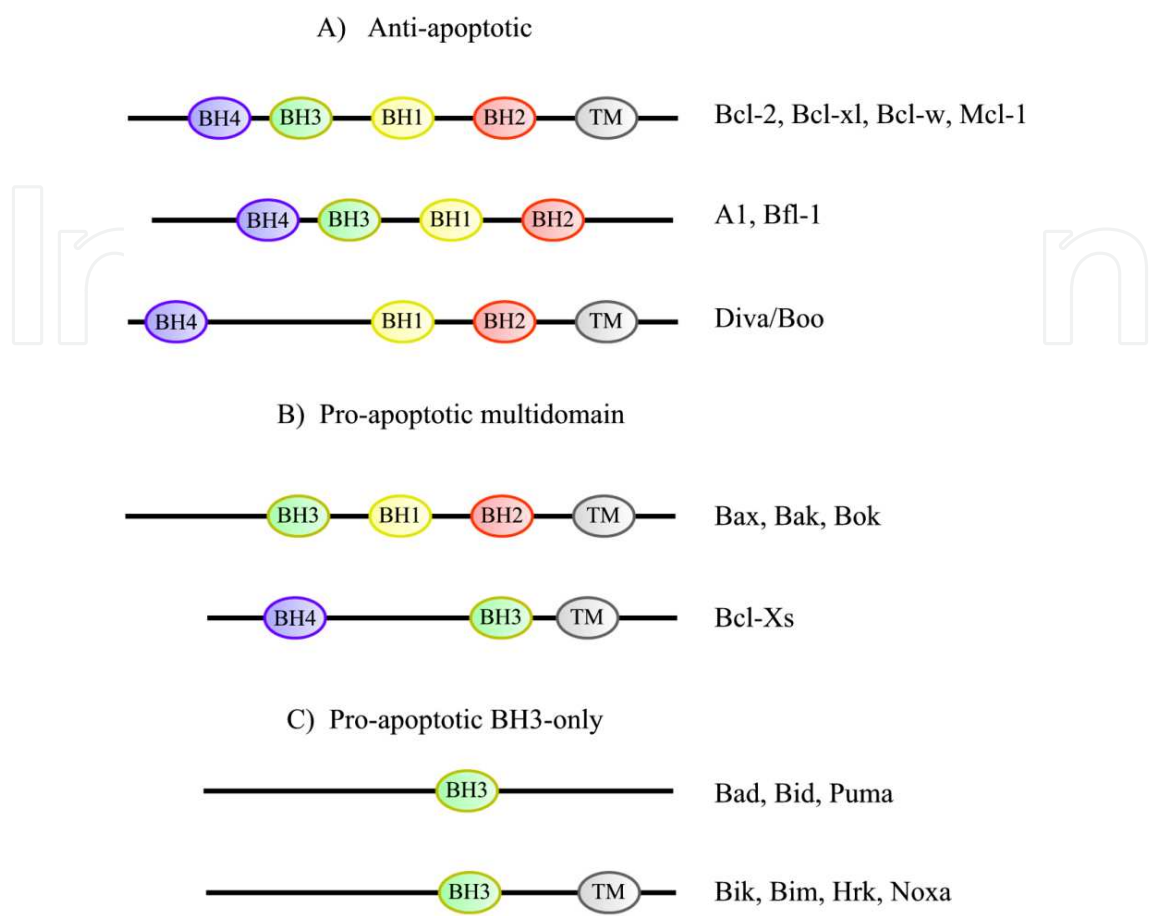


Figure 3. B-cell lymphoma 2 (Bcl-2) family proteins. A) General schematization of the structure of Bcl-2 proteins. B) The anti-apoptotic members – they possess all the four BH domains. C) The pro-apoptotic members which in turn are divided into two groups: multidomain and BH3 only.

its receptor, a series of intracellular complexes are formed to activate the initiator caspases (such as -8 and -10), which then activate the executioner caspases (such as -3, -6, and -7). In their activated form, these executioner caspases cleave multiple intracellular targets.

The intrinsic apoptotic pathway, in contrast, can be activated by various stimuli, including DNA damage, growth factor starvation, and oxidative stress [13]. During exposure of cells to these stimuli, the mitochondria are affected, since several members of the Bcl-2 family are activated and promote mitochondria outer membrane permeabilization (MOMP). The permeated external mitochondria membrane allows the release of cytochrome c (cyt c), which is associated with the Apaf-1 protein. The cyt c and Apaf-1 union then bonds to the initiator caspase-9 to form the complex that constitutes the apoptosome, which has the ability to activate the initiator caspases that perform their functions by cleaving specific cellular substrates.

The second process of cell death, autophagy, is a genetically programmed and evolutionarily conserved process that produces the degradation of obsolete organelles and proteins. It is activated by such extracellular stimuli as nutrient starvation, hypoxia, high temperature, and

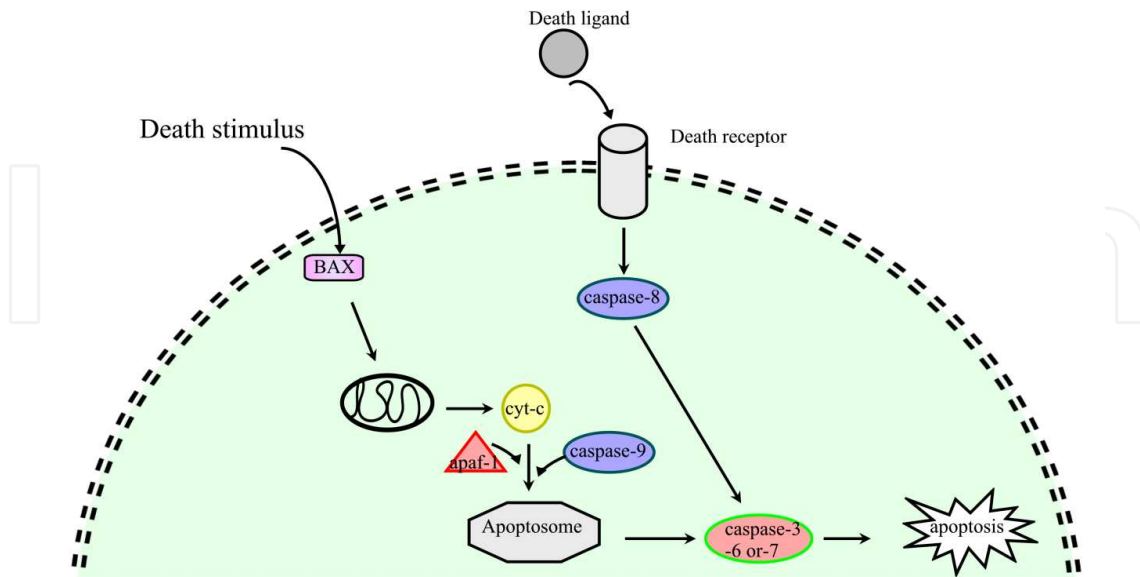


Figure 4. Routes of activation of apoptosis. The extrinsic route is mediated by external signals – a ligand – that activate to the membrane receptor. The ligand–receptor interaction induces the assembly of the death-inducing signaling complex (DISC) to promote the activation of caspase-8, which in turn is able to activate to the executor caspase -3, -6, or -7, conducting to the morphological changes of the apoptosis. The intrinsic route is directed by the mitochondrial outer membrane permeabilization, which allows the release of pro-apoptotic elements as cytochrome-C. Cytochrome-C induces the apoptosis protease-activating factor 1 (Apaf-1) to promote the activation of caspase-9 to assemble the apoptosome. The apoptosome is capable of activating to the executor caspases.

altered intracellular conditions, including the accumulation of damaged or superfluous organelles (reviewed in [2]).

In eukaryotic organisms, three types of autophagy have been described: microautophagy, macroautophagy (commonly called simply autophagy), and chaperone-mediated autophagy (Figure 5). Microautophagy involves the engulfing of cytoplasmic components directly at the level of the lysosome by means of an invagination process, while macroautophagy entails the formation of double-membrane vesicles that contain cellular components, which fuse with lysosomes to form an autophagolysosome. It is inside the autophagolysosome that the intravesicular components are degraded and, if possible, recycled by the cell (reviewed in [2 and 14]). Chaperone-mediated autophagy, finally, entails the participation of chaperones in recognizing the proteins designated for elimination by the lysosomes [14].

Autophagy is directed by *Atg* (AuTophagy-related) genes, which are required to activate the signaling complex that triggers the formation of autophagosomes [15]. *Atg* genes were discovered in yeast, but many have orthologues in higher eukaryotes (Figure 6). Autophagosome formation entails the participation of the cytoplasmic protein LC3 (*Atg8*), which undergoes lipidation by phosphatidylethanolamine, and is then recruited to the nascent autophagosome membrane (Figure 7). Accumulation of lipidated LC3 protein (known as LC3-II) is used as a marker of autophagy [16].

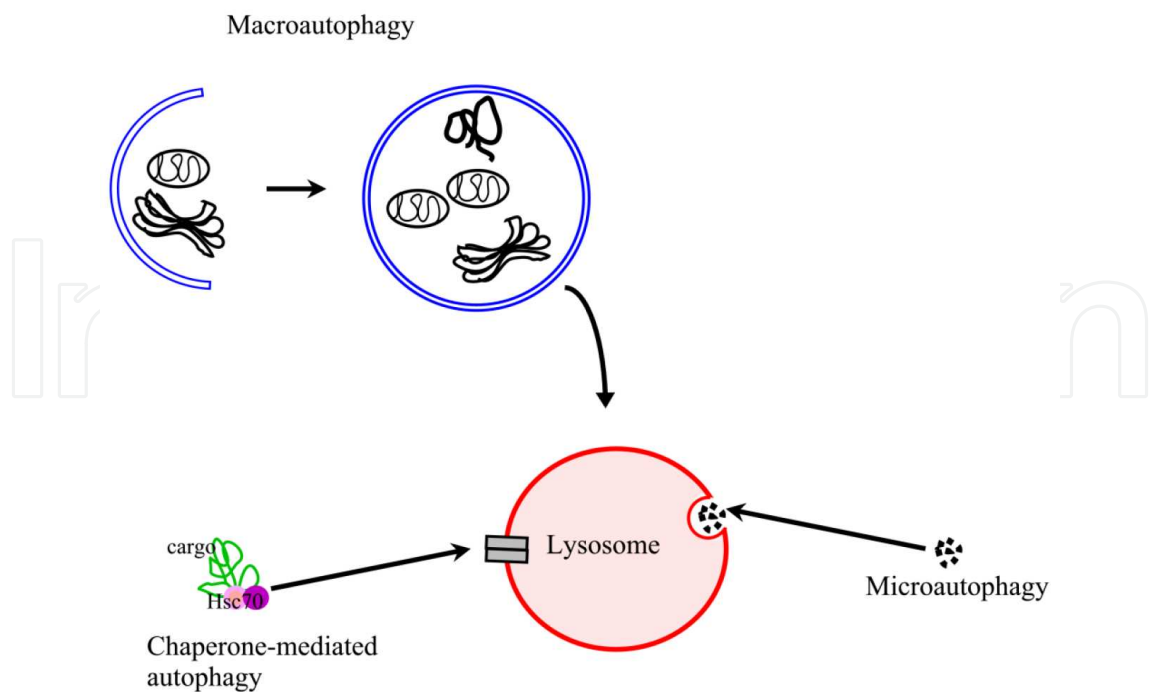


Figure 5. Schematic representations of the different mechanisms of autophagy. Macroautophagy – autophagy- implies the formation of a double-membrane vesicle, which engulfs cytoplasmic content that will be conducted to the lysosome to be degraded. Microautophagy is characterized by direct engulfing of cytoplasmic components by the lysosome. This process involves the remodeling of the membrane of the organelle by forming a lysosomal membrane invagination. During chaperone-mediated autophagy, the proteins to be degraded are targeted for an Hsp70, which in turn transport the target cargo to the lysosome.

Atg genes		
Nucleation	Expansion	Fusion
Atg 6 Atg 9 Atg 11 Atg 13 Atg 14 Atg 17 Vps 15 Vps 34	Atg 1 Atg 2 Atg 3 Atg 4 Atg 5 Atg 8 Atg 10 Atg 18	Vam 3 Vam 7 VTI 1 YKT 6

Figure 6. Atg protein family includes more than 30 members that participate in the different events that constitute the autophagic process.

Autophagic cell death, or type II programmed cell death, is characterized by a massive engulfing of the cytoplasm by autophagic vesicles. This intense autophagic activity differs substantially from autophagy that occurs continuously at basal levels. Ultrastructural studies in *Drosophila* have revealed the accumulation of autophagic vacuoles in most larval tissues.

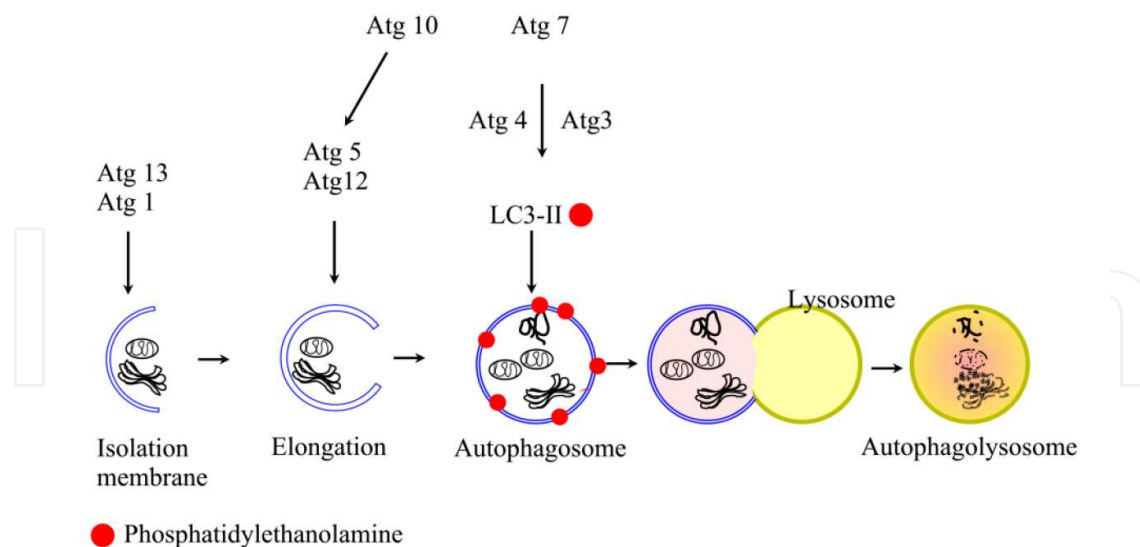


Figure 7. The formation of autophagic vacuoles involves the participation of different Atg proteins since the initial phases until the sequestration of cytoplasmic content. LC3 protein is lipidated by the phosphatidylethanolamine.

This type of programmed cell death begins with the degradation of cytoplasmic organelles by autophagy, though the cytoskeletal elements are conserved until the late stages of the process (reviewed in [17]).

3. Morphological characteristics of the necrosis process

Cell death caused by necrosis is considered an accidental, unprogrammed event that occurs under total ATP depletion [3], and that results from such external stimuli as extreme physical–chemical stress, heat, osmotic shock, mechanical stress, freezing, thawing, and high concentrations of hydrogen peroxide.

Necrotic cell death is characterized morphologically by generalized swelling of cell membranes, often accompanied by chromatin condensation and an irregular DNA degradation pattern [18]. The cytoplasmic membranes and membranous organelles dilate, and the increased cellular swelling causes the breakdown of the plasma membrane, which releases the cytoplasmic contents into the extracellular space (Figure 8). The release of the intracellular contents leads to massive cellular damage that affects neighboring cells, which explains why necrosis triggers inflammatory and autoimmune reactions. The necrosis process takes place in the absence of phagocytosis, and its final phase is characterized by the loss of the integrity of the cellular membrane. The release of the contents of necrotic cells includes molecules which act as signals that promote inflammation.

The most significant difference between programmed cell death (*i.e.*, apoptosis and autophagy) and necrosis is plasma membrane leakage and the consequent induction of inflammation in the affected tissue caused by the release of intracellular components [19].

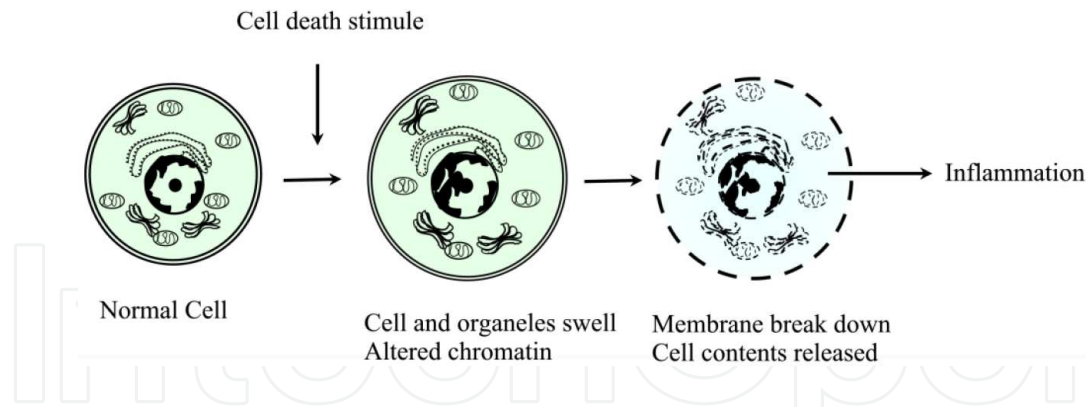


Figure 8. Morphological characteristics of necrosis involve the membranous swelling of the organelles, DNA degradation, and finally the release of the cytoplasmic content that affects the neighbor cells, provoking an inflammatory response.

4. Biochemical events during necrotic cell death

Despite these findings, however, necrosis is still considered an accidental process in which several factors exert effects on the cells to begin the elimination event. Cellular stress factors like low oxygen (hypoxia), cytokines, ischemia (restricted blood supply), heat, irradiation, pathogens, and toxin exposure can all lead to necrosis. These stimuli provoke several changes at the cellular level. While reactive oxygen species are produced by the mitochondria as a normal process, under pathological conditions, reactive oxygen molecules increase and induce damage in the biomolecules, which leads the cells toward necrosis. During necrosis, the levels of both reactive oxygen species and intracellular calcium increase (reviewed in [20]). It is important to consider that the internal cell environment is highly regulated, so certain stimuli are able to alter cell membrane permeability and thus produce an imbalance among different ions, such as potassium, sodium, and calcium. Calcium is regulated by the endoplasmic reticulum, and a loss of calcium homeostasis can lead to several intracellular alterations. In contrast, increased calcium levels can affect diverse mitochondrial functions and result in alterations of the production of reactive oxygen species. When high calcium levels are sustained over time, they disrupt mitochondrial inner membrane integrity and cause a loss of the ability to generate ATP [21] and, eventually, necrotic cell death (Figure 9). In addition to their effects inside the mitochondria, altered cytosolic calcium levels can activate different types of proteases, including calpains. Calpains are intracellular cysteine proteases present in inactive form, that may be activated by increased cytosolic calcium [22, 23]. Once activated, they can disrupt the lysosomal membrane with the resulting release of cathepsins B and L [24]. This group of reactions causes destabilization of the final membrane system. Together, these alterations cause the cell to lose its membranes such that the cellular contents are released into the extracellular space.

The molecular hallmark of necrosis is drastic ATP depletion, which is believed to be the underlying cause of cell death. There is a metabolic disruption accompanied by energy depletion and loss of ATP that leads to cellular edema, while the mitochondria become round and swollen, the endoplasmic reticulum dilates, the lysosomes are disrupted, and the formation of plasma membrane protrusions called blebs is apparent [25].

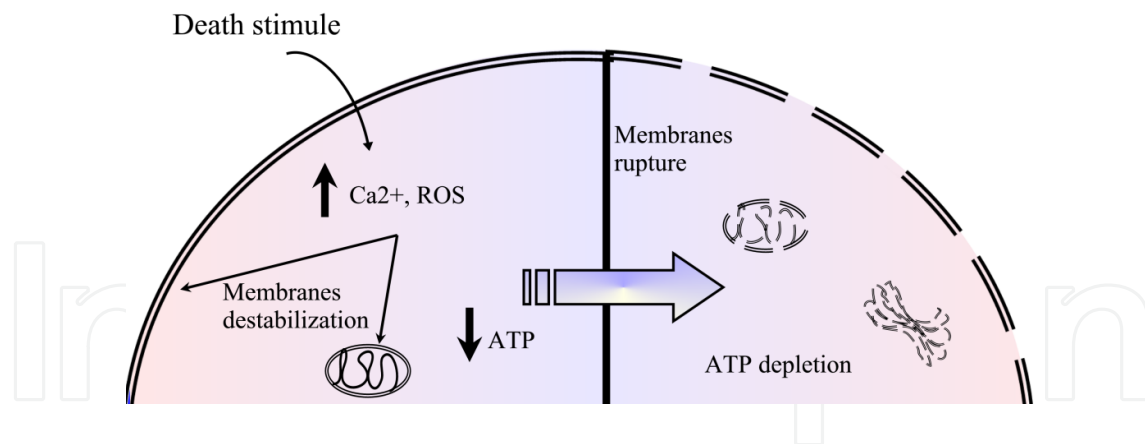


Figure 9. Biochemical cellular changes during necrosis cell death. The levels of both reactive oxygen species (ROS) as well as intracellular calcium (Ca^{2+}) are increased. Sustained high calcium levels alter cell permeability of the membranes, leading to the dysfunction and rupture of membranes. During necrosis, the ATP depletion is conducted.

Necrosis cell death occurs due to a failure in plasma membrane permeability that disrupts the plasma membrane and releases cellular components. This cell death process is associated with the indiscriminate extracellular release of soluble intracellular constituents through the permeabilized plasma membrane.

5. The emergence of necrosis as programmed cell death (necroptosis)

The way in which necrosis occurs allows us to determine that it is not merely accidental. Several examples of the presence of necrosis in different phases of an organism's development suggest that this process may be regulated during the embryonic stage. Necrosis is present during the longitudinal growth of bones in young animals [26], and is also found in intestinal epithelial cells in adults [27]. On the other hand, during caspase inhibition, necrosis may be activated as an alternative route of cell elimination, suggesting that it is not simply an accidental process, but that, under certain conditions could function as a programmed event called necroptosis.

The necrosis process has long been conceived as an accidental, passive event; however, recent detailed observations were able to identify dying cells by the expression of different proteins and the intracellular disposition of several proteins that may be active during the event. As mentioned above, programmed cell death, or apoptosis, can be activated by two routes, one of which entails the participation of receptors present in the cytoplasmic membrane. These receptors include $\text{TNF}\alpha$, FasL, and TRAIL that, once activated, cause recruitment of a protein complex (death-inducing signaling complex, DISC) mediated by the adaptor protein FADD (Fas-associated protein with death domain) to activate the initiator caspase-8 [28]. This activation of the caspase system triggers execution of the apoptotic process. Diverse findings indicate that the receptors involved in apoptotic cell death may also participate in the occurrence of a different type of cell death under distinct conditions of molecular resource availability. For cells that do not express caspase-8, it was predicted that they could not respond to an apoptotic stimuli directed by FasL induction. However, those results were surprising

because they included the multimerization of FADD in the absence of caspase activation. The morphology of apoptosis was not present, but ultrastructural analyses of those dying cells revealed necrotic morphological changes [29]. All these observations suggest that the receptor regulators of apoptosis were involved not only in that process, but also in the activation of a different signaling pathway that allows the formation of protein complexes which lead the cell toward a death process with necrotic features. These developments led to the emergence of a new concept of programmed cell death called necroptosis, whose morphological characteristics are similar to those of accidental necrosis, although the molecular events that occur indicate that it is a coordinated process. Necroptosis has been found under special conditions, where pro-apoptotic enzymes were absent or limited. In experimental embryo models, interdigital membrane regression in mouse embryos was effectuated by necrosis triggered by either caspase inhibition or drugs [30]. Necroptosis is thus a form of programmed cell death that has been demonstrated under experimental conditions, when apoptosis is inhibited.

6. Biochemical aspects of necroptosis

Several studies have succeeded in discerning the molecular events that occur during necroptosis, and it is those events that differentiate between necrosis (an accidental process) and necroptosis (a programmed process). Necroptosis has been observed in several pathological cell death events, such as ischemic brain injury, myocardial infarction, exotoxicity, and chemotherapy-induced cell death [31].

Necroptosis is morphologically characterized by several cytoplasmic changes. In fact, it is sometimes possible to distinguish the different degrees of advance of this process as the organelles swell, the cell membrane fragments, and cytoplasmic and nuclear disintegration become evident. During necroptosis, the nuclei remain intact and there is no massive caspase activation, chromatin condensation, spillage of cell contents, phagocytosis by macropinocytosis, lysosomal leakage, or oxidative bursts [32]. The term necroptosis has been introduced to identify a process of cell death with morphological characteristics distinct from those of apoptosis. Because there was no caspase activation during this process, it is called “caspase-independent”.

Necroptosis is a programmed event that ends with the delivery of the cytoplasmic contents into the extracellular space. Membrane destabilization is a consequence of different intracellular mechanisms that generate osmotic changes by damaging the ion balance. When DNA damage occurred due to reactive oxygen species, the PARP protein was activated and began the reparation process; however, this process consumed abundant ATP and that reduction initiated a sequence of events that led to a deficient cellular efflux of calcium. The decreased ATP levels affected the activity of Na⁺-K⁺ ATPase, which requires a large amount of ATP in order to function correctly. This decreased Na⁺-K⁺ ATPase activity reduced calcium release and, as a result, increased intracellular calcium levels, leading to membrane destruction (reviewed in [33]). The breakdown of the cellular membrane, in turn, released several signals

that activated the immune system. These soluble signals were proteins with pro-inflammatory properties that stimulate the recruitment of neutrophils to the site of cell death [34].

The mechanism proposed for the onset of necroptosis involves participation of the TNF-R (tumor necrosis factor-receptor), Fas, and TRAIL receptors, all of which belong to the tumor necrosis factor/nerve growth factor receptor superfamily and are involved in apoptotic cell death (Figure 10). Activation of TNF receptors by their ligands triggers different responses that involve pro-survival or pro-cell-death processes. Activated TNFR1 induces recruitment of TRADD, TRAF2/2, RIPK1, IAPs, and LUBAC to form a pro-survival complex that activates NF-kappaB, JNK, and p38 MAPKs (reviewed in [35]). However, once this complex becomes established it is able to recruit FADD and procaspase-8, which produces a complex that could initiate either apoptosis or necroptosis. Under conditions of low levels of procaspase-8, a different complex is formed, – one that includes the receptor which interacts with protein 1 (RIP1 – a serine/threonine kinase activator –) and leads to the onset of necroptosis cell death. Biochemically, necroptosis is defined as a form of cell death that is dependent on RIP1, which is the target protein in necrotic cell death induced by the TNF α , TRAIL, and CD95 receptors [36].

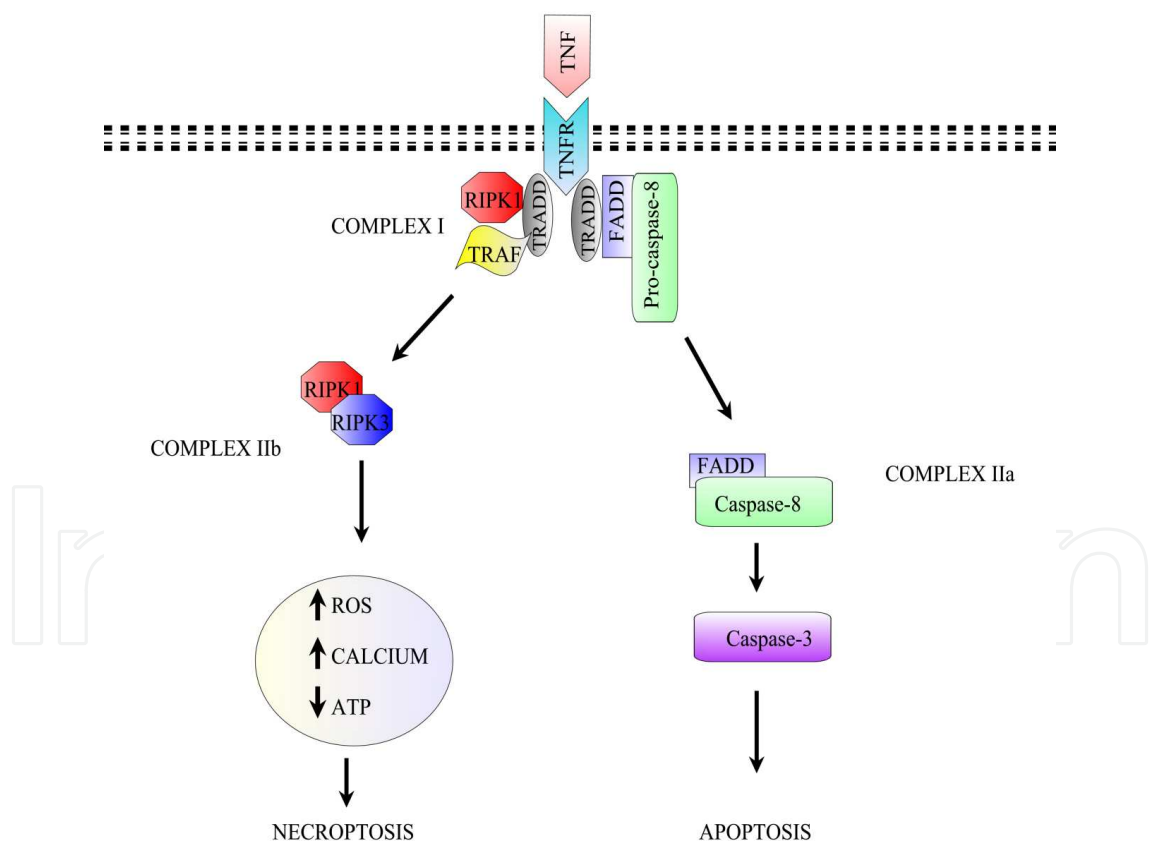


Figure 10. Biochemical aspects of necroptosis. TNFR recruits TRADD; this recruitment allows the formation of different complexes related to the RIPK1 protein or to the pro-caspase-8. The TRADD-FADD-pro-caspase-8 allows caspase-8 activation, which in turn activates the executor caspases, promoting the apoptosis process. Under conditions where caspase-8 is inhibited, the formation of the complex TRADD-FADD-RIPK1 initiates necroptosis.

The RIP3 is implicated in necroptosis during inflammatory responses to virus infections [37], and during cellular necrosis in response to the TNF- α family of death-inducing cytokines [38]. RIP3 mediates necroptosis induced by Smac mimetic and TNF α [38].

Activation of RIP can be directed not only by TNFR, but also by other death receptors, such as Fas. Activation of ligands associated with apoptotic cell death, such as Fas, in conditions that are unfavorable for apoptosis, that is, when caspases are absent or inhibited, allows RIP activation, which leads to death by necrosis [36, 39].

The biochemical process of necroptosis is a new and active field, so not all the routes of activation of this event have been determined. Diverse studies concur that kinase RIP is involved, as we have mentioned. Another protein, PARP-1 (poly (ADP-ribose) polymerase-1), has been shown to be involved in necrotic cell death by means of DNA-damaging agents [40], since PARP-1 is an abundant repair nuclear protein. PARP-1 is activated via TRAIL-induced necroptosis that induces ATP depletion [41]. DNA strand breaks promote the activation of PARP-1 (poly(ADP-ribose) polymerase-1) for DNA repair; PARP-1 binds to DNA strand breaks using NAD⁺ as substrate, generating a negatively charged PARP-1, which in turn is dissociated from DNA ends, allowing the DNA repair process [42, 43]. Intensive PARP-1 activation can generate increased NAD⁺ depletion and, as a result, an energy failure that leads to necrosis. In neuronal cells under severe oxidative stress, PARP-1 activation resulted in NAD⁺ and ATP depletion that caused cell death [44].

7. Concluding remarks

Cell death is a normal event that controls tissue homeostasis. Today, we know that cells can be eliminated by means of different pathways that involve programmed or accidental mechanisms. Apoptotic cell death has been considered the major factor in physiological cell death, but recent evidence demonstrates that other routes of cell elimination – such as autophagy – also play important roles in maintaining homeostasis. A third route of cell death is necrosis, which was long considered an accidental form, characterized by general membrane swelling and ATP depletion. More recently, however, a new concept has been introduced: necroptosis. Necroptosis has been proposed as a kind of programmed cell death that is distinct from necrosis and apoptosis; one in which several signals involved in apoptosis participate significantly to initiate the process. It is important to note that necroptosis is an event that can be activated and regulated by such receptors as TNF or Fas – both of which are involved in the extrinsic route of apoptosis activation – when the pro-apoptotic signals are not available or are inhibited.

Activation of death receptors triggers a signaling cascade that includes activation of kinase RIP1, which in turn generates diverse intracellular reactions that lead the cell toward energy failure and conclude with the loss of intracellular homeostasis and the rupture of the membranes that, finally, generates an immunological response.

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